

## **The nucleolus – a gateway to viral infection?**

### Brief Review

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**Summary.** A number of viruses and viral proteins interact with a dynamic sub-nuclear structure called the nucleolus. The nucleolus is present during interphase in mammalian cells and is the site of ribosome biogenesis, and has been implicated in controlling regulatory processes such as the cell cycle. Viruses interact with the nucleolus and its antigens; viral proteins co-localise with factors such as nucleolin, B23 and fibrillarin, and can cause their redistribution during infection. Viruses can use these components as part of their replication process, and also use the nucleolus as a site of replication itself. Many of these properties are not restricted to any particular type of virus or replication mechanism, and examples of these processes can be found in DNA, RNA and retroviruses. Evidence suggests that viruses may target the nucleolus and its components to favour viral transcription, translation and perhaps alter the cell cycle in order to promote virus replication. Autoimmunity to nucleolin and fibrillarin have been associated with a number of diseases, and by targeting the nucleolus and displacing nucleolar antigens, virus infection might play a role in the initiation of these conditions.

### **Introduction**

The eukaryotic nucleus contains a number of domains or subcompartments, which include nucleoli, nuclear Cajal bodies, nuclear speckles, transcription and replication foci, and chromosome territories [34]. For many years the exclusive function of the nucleolus was thought to be ribosomal rRNA synthesis and ribosome biogenesis. Recently, however, the nucleolus has been implicated in many aspects of cell biology that include functions such as gene silencing, senescence, and cell cycle regulation [8, 50, 51].

The nucleolus is the site where 5.8S, 18S and 28S rRNAs are transcribed, processed, and assembled into ribosome subunits [52]. The nucleolus is composed

of (or contains) many different factors including: nucleolin, fibrillarin, spectrin, B23, rRNA, and ribosomal proteins S5 and L9 [63, 65]. Electron microscopy revealed that the nucleolus consists of at least three different regions; fibrillar centres, a dense fibrillar component and a granular component [63]. These regions may have different functions. For example, the perinucleolar compartment has been implicated in RNA metabolism [29].

### **Nucleolar antigens**

Three of the most abundant and well-understood proteins in the nucleolus are nucleolin, fibrillarin and B23. Nucleolin (first called C23), represents approximately 10% of the total nucleolar protein content and is highly phosphorylated, methylated, and also can be ADP-ribosylated [25]. Nucleolin has the potential to bind to multiple RNA targets and this may reflect its variety of functions [25, 26]. One of the main functions of nucleolin is facilitating the first cleavage step of rRNA in the presence of U3 snoRNP. Nucleolin may function as a chaperone for correct folding in pre-rRNA processing [27]. Nucleolin has also been implicated as a repressor of transcription [78]. Whilst mammalian nucleolin has a predicted molecular mass of approximately 77 kDa (depending on the species), the apparent molecular mass is between 100 and 110 kDa, and has been attributed to the amino acid composition of the N-terminal domain, which is highly phosphorylated [25].

Fibrillarin has a molecular mass of approximately 35 kDa and is highly conserved in sequence, structure and function in eukaryotes, and analysis indicated that human fibrillarin has a potential RNA binding domain in its central part [3]. Fibrillarin is directly involved in many post-transcriptional processes including pre-rRNA processing, pre-rRNA methylation, and ribosome assembly [75].

B23 (also called numatrin, nucleophosmin or NO38) is widely distributed amongst different species with approximately the same molecular mass of 35–40 kDa [44, 65]. Two isoforms of the protein are expressed, the major form (B23.1) is predominately located in the nucleolus and the minor form (B23.2) is located in the cytoplasm. Similar to nucleolin and fibrillarin, B23 is likely to have multiple functions and has been implicated in ribosome assembly [17], binding to other nucleolar proteins, nucleocytoplasmic shuttling [37] and possibly regulating transcription of rDNA by mediating structural changes in chromatin [49].

### **The nucleolus and the cell cycle**

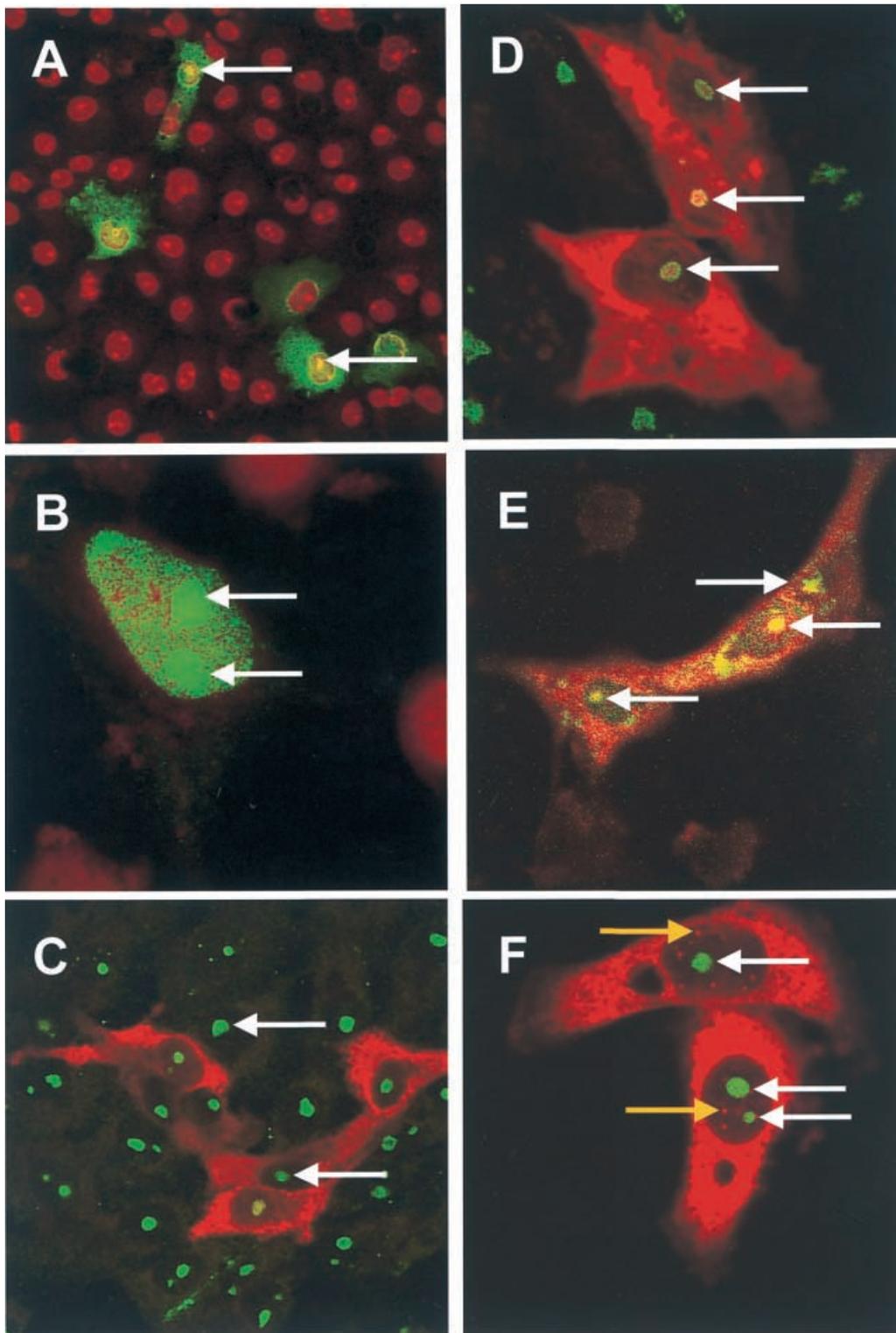
The nucleolus and associated proteins are also implicated in (and regulated by) the cell cycle [8]. During interphase in higher eukaryotic cells the number of nucleoli vary depending on the stage of the cell cycle, and the nucleolus disappears at the start of mitosis [2]. During G<sub>1</sub> cells can contain more than one nucleolus. This is probably reflected in the fact that these cells are translationally active, and therefore require more ribosomes, whose synthesis may in turn be controlled by the phosphatidylinositol-3-OH kinase (PI(3)K) pathway [74]. As the cells progress through S phase and into G<sub>2</sub>, where single nucleoli can be present. The nucleolus then disperses during mitosis. At telophase nucleogenesis involves

the distribution of material derived from the mother cell toward the active NORs of the daughter nuclei and form pre-nucleolar bodies (PNBs) [19]. Subsequent nucleolar reformation is a dynamic and complex process [4]. Cajal bodies associate with nucleoli, and contain many similar components such as fibrillarin [55, 69], and they sequester CDK2 and cyclin E in a cell cycle dependent manner [40]. The concentration of nucleolin and B23 [68] and the distribution of fibrillarin are dependent on the cell cycle [4, 23]. Nucleolin is stable in proliferative cells, but undergoes self-cleavage in quiescent cells [11] (e.g. Fig. 2, lane 1) and has been suggested to be involved in regulation of cell growth and proliferation [70].

**Table 1.** Examples of viral proteins that localise to the nucleolus and sub-nuclear compartments

Virus	Protein	Nucleolus	Interaction with nucleolar antigens	Reference(s)
<i>RNA viruses</i>				
BDV	Replication complex	+		[57]
BVDV	Capsid	+		[61]
CMV	3A	+		[42]
	Capsid	+		[38]
Coronavirus	Nucleoprotein	+	+	[28, 77]
HDV	Delta antigen	+	+	[35]
	Viral RNA			
Influenza A virus	NP	+		[15, 16]
NDV	Matrix protein	+		[53]
Poliovirus	(3' NCR)		+	[76]
PRRSV	Nucleoprotein	+		[62]
SFV	Capsid protein	+		[22]
<i>Retrovirus</i>				
HIV-1	Rev	+	+	[18, 21]
	Tat	+		[67]
HTLV-1	Rex	+	+	[1, 66]
<i>DNA viruses</i>				
Adenovirus	IVa2	+		[41]
	V	+	+	[45, 46]
EBV	EBNA5	+		[72]
HSV-1	Us11	+		[43]
	ICP27	+		[47]
MDV	MEQ	+		[39]

Abbreviations: BDV (Borna disease virus), BVDV (bovine viral diarrhoea virus), CMV (cucumber mosaic virus), EBV (Epstein Barr virus), HDV (hepatitis delta virus), HIV (human immunodeficiency virus), HSV (herpes simplex virus), HTLV (human T-cell leukaemia virus), MDV (Marek's disease virus), NDV (Newcastle disease virus), PRRSV (porcine reproductive and respiratory syndrome virus) and SFV (Semliki Forest virus)



### Viral interactions with the nucleolus

As a consequence of infection or a deliberate process, a number of viruses interact with the nucleolus and its components. It is the site of Borna disease virus replication and transcription [57], and might be involved in HIV-1 RNA processing [48]. A number of viral proteins localise to the nucleolus, with examples from animal retroviruses and DNA viruses, and animal and plant RNA viruses (Table 1). Why viral proteins localise to the nucleolus has not been precisely determined, although given the multifunctional role of the nucleolus several activities could be targeted, including cellular transcription [39, 56], virus transcription [46], virus translation [28] or cell division [77].

Nucleoli can be visualised using immunofluorescence by mounting preparations in propidium iodide to visualise nuclear DNA and regions of rRNA synthesis [28] or using antibodies to nucleolar antigens [46, 77], or transfecting cells

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**Fig. 1.** **A** Detection of bovine viral diarrhoea virus (BVDV) capsid protein (green) by indirect immunofluorescence and nuclear DNA (red) by direct fluorescence using a confocal microscope in cells transfected with a plasmid expressing BVDV capsid protein with a C-terminal V5 epitope under the control of a CMV promoter. V5 epitope was detected using mouse monoclonal anti-V5 antibody and nuclear DNA and regions of rRNA transcription (nucleoli) were visualised by staining cells with propidium iodide. Nucleoli are visible as bright red dots, yellow indicates colocalisation and examples of nucleoli in transfected cells are arrowed. **B** Detection of influenza A virus nucleocapsid protein (NP) (green) by indirect immunofluorescence and nuclear DNA (red) by direct fluorescence using a confocal microscope in cells transfected with a plasmid, pCDNA3-NP, expressing NP (generously provided by Dr. Wendy Barclay [71]). NP was detected using mouse monoclonal anti-NP antibody and nuclear DNA was visualised by staining cells with propidium iodide. Nucleoli are arrowed. **C** Detection of infectious bronchitis virus nucleoprotein (N protein) (red) and B23 (green) by indirect immunofluorescence using a confocal microscope in cells transfected with a plasmid expressing IBV N protein under the control of a CMV promoter (pCi-IBV-N) [28]. N protein was detected with rabbit anti-IBV polyclonal sera (generously provided by Dr. Dave Cavanagh) and B23 with anti-B23 (Human) goat polyclonal antibody (Santa Cruz Laboratories). Examples of nucleoli are arrowed. **D** Detection of murine hepatitis virus (MHV) N protein (red) and fibrillarin (green) by indirect immunofluorescence using a confocal microscope in cells transfected with a plasmid expressing MHV N protein under the control of a CMV promoter (pCi-MHV-N) [77]. MHV N protein was detected with rabbit anti-MHV polyclonal sera (generously provided by Prof. Peter Rottier) and fibrillarin with anti-fibrillarin (Human) mouse monoclonal antibody (Cytoskeleton Research). Yellow indicates colocalisation. Examples of nucleoli are arrowed. **E** Detection of MHV N protein (red) by indirect immunofluorescence and a fibrillarin/GFP fusion protein (green) by direct immunofluorescence using a confocal microscope. Cells were co-transfected with pCi-MHV-N and a plasmid expressing a fibrillarin/GFP fusion protein (generously provided by Prof. Angus Lamond). MHV N protein was detected with rabbit anti-MHV polyclonal sera. Yellow indicates colocalisation. Nucleoli are arrowed. **F** Detection of MHV N protein (red) and nucleolin (green) by indirect immunofluorescence using a confocal microscope. Cells were transfected with pCi-MHV-N. MHV N protein was detected with rabbit anti-MHV polyclonal sera and nucleolin with mouse anti-human nucleolin monoclonal sera (Leinco Laboratory). Nucleoli are indicated by white arrows and examples of nuclear speckles by yellow arrows

with plasmids that express nucleolar antigens tagged with fusion proteins such as green fluorescent protein (GFP) [55, 69]. Several examples of these techniques in conjunction with viral proteins that localise to the nucleolus are shown in Fig. 1.

### **Viruses can redistribute and interact with nucleolar antigens**

Virus infection can also result in the redistribution, or viral proteins become associated with, nucleolar antigens (Table 1). For example, an adenovirus infection results in the redistribution of nucleolin and B23 [46] and Okuwaki et al. [49] have shown that B23 stimulates adenovirus replication. Nucleolin is prevented from entering the nucleus in poliovirus-infected cells [76]. Nucleolin has been shown to interact with the poliovirus 3' non-coding region (NCR) and as a result, was suggested to be involved in replication [76]. In addition, nucleolin was shown to stimulate IRES-mediated translation of the poliovirus genome [30]. Nucleolar targeting and an interaction with nucleolin has been shown to promote hepatitis delta virus (HDV) replication [35]. Adenovirus [56] and coronavirus [13] infection results in the redistribution of fibrillarin. By altering the distribution of fibrillarin, viruses might be reducing polII transcription i.e. the synthesis of rRNA, as blocking fibrillarin with antibody prevented its translocation to nucleoli and resulted in the reduction or inhibition of polII transcription [23]. Interestingly PolII transcription is disrupted in adenovirus-infected cells [10]. Nucleolin (and proteins belonging to the nucleolin super-family) have been suggested to act as possible cell surface receptor for coxsackie B viruses [58] and HIV [7], and a nucleolin-gag interaction may be involved in the assembly of Moloney murine leukaemia virus [5].

### **Other sub-nuclear structures associated with the nucleolus are also targeted during viral infections**

Viral proteins also target other sub-nuclear bodies, such as Cajal (coiled) bodies (e.g. Marek's disease virus (MDV) MEQ protein [39]), nuclear speckles (e.g. HIV-1 Rev protein [32] and mouse hepatitis virus nucleoprotein Fig. 1F) and nuclear domain 10 (ND-10s – also known as promyelocytic leukaemia-associated nuclear body (PML)) (e.g. adenovirus 5 E1b 55K, E1A and E4-ORF3 proteins [9, 36]). ND-10s have been implicated in the modulation of the interferon response [79] and by targeting this structure viruses have been shown to modulate downstream antiviral effects (e.g. arenavirus infection [6]).

### **Factors effecting nucleolar localisation**

A number of factors can determine whether a protein localises to the nucleolus. Soluble proteins of less than 40–60 kDa can diffuse passively into the nucleoplasm through the nuclear pore complex, and could in principal diffuse in and out of the nucleolar compartment [54, 59]. Non-specific RNA binding proteins that diffuse into the nucleus may therefore be expected to become concentrated in the nucleolus because a large amount of rRNA is present. The transport of larger proteins through the nuclear pore is an active process requiring ATP and nuclear

localisation signals (NLSs), which also make up (in part) nucleolar localisation signals (NuLS). NLSs include the ‘pat4’ motif, which consists of a continuous stretch of four basic amino acids (arginine and lysine), and the ‘pat7’ motif, which starts with a proline and is followed within three residues by a segment containing three basic residues out of four [24], or a bipartite signal [60]. Localisation of a protein to the nucleolus is probably a result of targeting to the nucleus via NLSs followed by an interaction between the target molecules (via the NuLS) and components that make up the nucleolus [8, 65]. An example of a protein that localises to the nucleolus in this manner is nucleolin, which contains a bipartite NLS and associates with rRNA in the nucleolus via RNA binding domains [64].

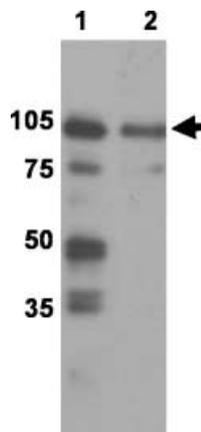
Some viral proteins that localise to the nucleolus can also be found in the nucleoplasm, e.g. MDV MEQ [39] suggesting some viral proteins localise to the nucleolus do so by diffusing through the nuclear pore, into the nucleoplasm, and associating with nucleolar factors in the nucleolus. Alternatively viral proteins might stay associated with nucleolar antigens such as nucleolin and fibrillarin, which are exchanged between the nucleoplasm and the nucleolus [12].

Several viral NuLS have been identified (Table 2), either by sequence comparison to known NuLSs or experiments where candidate NuLSs have been used to target fusion proteins to the nucleolus (e.g. arteriviruses [62]). Viral NuLSs can be characterised by possessing either ‘pat4’ or ‘pat7’ motifs (e.g. the coronaviruses

**Table 2.** Amino acid sequence analysis of examples of virus proteins that contain nucleolar localisation signals (NuLS) or signals that mediate an interaction with nucleolin

Protein	(Amino acid position)	NuLS	Reference
Adenovirus protein V	23–42	<u>KKEEQDYKPRKLRVKKKKK</u>	[46]
	315–337	<u>RPRRRATTRRRTTTGTRRRRRRR</u>	
	159–182	<u>KRGLKRESGDLAPTQQLMVPKRQRL</u>	
BVDV capsid protein	71–91	<u>HNKNKPPESRKKLEKALLAW</u>	[61]
HDV antigen	35–50	<b>RKLKKKIKKLEEDNPWC</b>	[35]
	51–65	LGNIKGIIG <b>KKDK</b> DGC	
IBV nucleoprotein	350–369	<u>GNSPAPRQQRPKKEKLLKKQ</u>	[28]
MDV MEQ protein	62–78	<u>RRRKRNRDAARRRRRKQ</u>	[39]
SFV capsid protein	73–90	<u>KPKKKKTTPKPKTQPKK</u>	[22]
	92–105	<u>KKKDKQADKKKKKP</u>	
TEGV nucleoprotein	331–350	<u>RPSEVAKEQRKRKRSKSAE</u>	[77]

Abbreviations used are detailed in Table 1, except IBV (infectious bronchitis virus) and TGEV (transmissible gastroenteritis virus). Potential NuLS are underlined. The nucleolin binding sites in HDV antigen are shown in bold face



**Fig. 2.** Western blot detection of nucleolin from Vero cell nuclear extracts (1), and nuclear extracts passed over infectious bronchitis virus nucleoprotein immobilized on NTA beads (2). Nucleolin was detected using C23 (human) goat polyclonal antibody (Santa Cruz Laboratories). The positions of molecular weight markers (kDa) are shown to the left. The position of mature nucleolin is arrowed

[28, 77]) or stretches of basic residues, for example adenovirus protein V [46] or MDV MEQ protein [39]. Interestingly, Matthews [46] identified a possible bipartite NuLS in adenovirus protein V (Table 2, amino acids 157–184). Some viral NuLS have been shown to control interaction with nucleolin, e.g. HDV large antigen, which contain two nucleolin binding domains (Table 2) and determine targeting of the protein to the nucleolus [35].

Infectious bronchitis virus (IBV) nucleoprotein also associates with nucleolin (Fig. 2) and HIV-1 Rev protein with B23 [21]. Rev and Rex proteins localise to the nucleolus and contain a potential NuLS [14, 33]. Rev protein colocalizes with B23 in the granular and dense fibrillar regions of the nucleolus [18], and rRNA synthesis may be critical for the nucleolar localisation of both proteins [18].

### Nucleolar antigens and auto-immunity

Auto-immunity to fibrillarin has been associated with the disease scleroderma [20, 73], and auto-immunity to nucleolin with systemic lupus erythematosus [31]. Viral antigens which localise to the nucleolus of infected cells and displace (or mimic) nucleolar antigens, might therefore stimulate the host immune response into producing antibodies to viral proteins that could have the potential to bind with the equivalent host proteins through structural mimicry. For example, poliovirus infection, which prevents nucleolin from entering the nucleus [76], may cause an increase in nucleolin in the cytoplasm and concomitant increase in cell surface expression. Thus, understanding the basis of viral protein interactions with the nucleolar milieu, and its consequences, may extend our understanding of how such autoimmune conditions develop.

### Conclusion

The nucleolus is fundamental to the control of many processes inside the cell including ribosome biogenesis, RNA processing, cell senescence, telomerase activity and the cell cycle, and is thus central to the normal operating of a cell. Interaction with the nucleolus is a pan-virus phenomena and data is accumulating

to suggest that viruses can use the nucleolus or its antigens to enhance viral replication, either by interacting directly with proteins such as nucleolin, or altering host cell transcription, translation and possibly the cell cycle. Apart from altering cellular functions, viral interactions with the nucleolus may contribute to the progression of autoimmune diseases associated with nucleolar antigens.

### Note added in proof

Since submitting this manuscript, Andersen et al. [Curr Biol (2002) 12: 1–11] have conducted a proteomic analysis of the nucleolus from HeLa cells and identified 271 nucleolar proteins.

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